

RAPID DETERMINATION
OF PROTEIN GLOBAL FOLDS

[0001] This application claims priority from prior copending provisional application serial no. 60/241,841, filed October 20, 2000.

BACKGROUND OF THE INVENTION

1. Technical Field

[0002] This invention relates to a protocol for the rapid determination of protein structure. In particular, the invention provides a method for rapidly obtaining protein or peptide structural information using only about 20-25% of the data set normally required in prior methods with a high degree of accuracy. The method allows the process to be automated to achieve results with a savings of time and labor.

2. Description of the Background Art

[0003] The determination of protein secondary, tertiary and quaternary structure is important in analyzing structural and functional relationships between all types of ligands and their receptors, enzymes and their substrates, or of any protein. For example, protein structure determination of a particular receptor generally can assist in rational drug design efforts to discover or synthesize more potent ligands at that receptor, or to design ligands with different activity profiles. Thus, existing pharmaceutical agents may be improved or changed to alter activity using detailed protein structural information. In

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addition, new chemical agents useful for treating disease can be developed using detailed structural information about receptors or other proteins in solution, receptors bound with ligand, or both.

[0004] X-ray crystallography is widely used to obtain detailed structural information about proteins and can provide the complete tertiary structure (global fold) of the backbone of a protein. This method, however, has several disadvantages. For example, only proteins which can be crystalized may be studied using X-ray crystallography. Some proteins are very difficult or impossible to crystalize. Moreover, crystalization can be very time consuming and expensive. Another major disadvantage of this method is that the structural information obtained is pertinent to the crystalline structure of the protein rather than the structure of the protein in solution. The bond angles present in a crystal structure may not be the same as those of the protein when it is in its active conformation and therefore may not provide information relevant to the biological or physiological system of interest.

[0005] Protein structure determination by high resolution multinuclear NMR also has become well known. In principle, this method gives all the information needed to determine the structure of a protein. Practically, however, the method is extremely time-consuming. In addition, in the past it has been very difficult to obtain accurate information about the structure of large proteins, for example 30-40 kilodaltons and especially 50 kilodaltons or larger using this method.

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[0006] Traditional methods for the determination of protein structure using NMR utilized distance data derived from NOE spectra. Very recently, residual dipolar couplings have become established as additional conformational restraints in the determination of the solution structures of proteins via high resolution multinuclear NMR. Tolman et al., *Proc. Natl. Acad. Sci. USA* 92:9270-9283, 1995; Tjandra et al., *J. Am. Chem. Soc.* 118:6264-6272, 1996; Tjandra and Bax, *Science* 278:1111-1114, 1997; Bax and Tjandra, *J. Biomol. NMR* 10:289-292. The introduction of a number of lyotropic dilute liquid-crystalline solutions and other methods for weak macromolecular alignment has enabled straightforward measurement of these couplings for a variety of macromolecules. See Bax and Tjandra, *J. Biomol. NMR* 10:289-292, 1997; Losonczi et al., *J. Biomol. NMR* 12:447-451, 1998; Prosser et al., *J. Am. Chem. Soc.* 120:11010-11011, 1998; Clore et al., *J. Am. Chem. Soc.* 120:10571-10572, 1998; Hansen et al., *Nature Str. Biol.* 5:1065-1074, 1998; Kiddle and Homans, *FEBS Lett.* 436:128-130, 1998; Wang et al., *J. Biomol. NMR* 12:443-446, 1998; Ottinger and Bax, *J. Biomol. NMR* 13:187-191, 1999; Fleming et al., *J. Am. Chem. Soc.* 122:5224-5225, 2000; Rückert and Otting, *J. Am. Chem. Soc.* 122:7793-7797, 2000.

[0007] Recently, interest has developed in the rapid determination of protein structural information based on residual dipolar couplings. Mueller et al. have developed a methodology for orienting peptide planes using dipolar couplings which determined the global fold of maltose binding protein in complex with β -cyclodextrin. This gave rise to pairwise RMSD (root mean square deviation) values between N-

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and C-terminal domains of the NMR structure and the corresponding regions in the X-ray structure of 2.8 Å and 3.1 Å, respectively. Mueller et al., *J. Mol. Biol.* 300:197-212, 2000; Mueller et al., *J. Biomol. NMR* 18:183-188, 2000. Lower values indicate less variation in the calculations and a more accurate structure. Generally, any value greater than 3 Å is considered quite inaccurate. Therefore, improvements in the variation would be greatly desired.

[0008] Fowler et al. (*J. Mol. Biol.* 304:447-460, 2000) have utilized $N_i-H_i^N$, $H_i^N-H_{\alpha i}$, $H_i^N-H_{\alpha i+1}^N$, $H_i^N-H_{i+1}^N$ residual dipolar couplings together with a small number of backbone-sidechain NOEs to determine the backbone fold of acyl carrier protein to an RMSD between backbone atoms of about 3 Å. Hus et al. (*J. Mol. Biol.* 298:927-936, 2000) have utilized long-range order restraints available from paramagnetic systems in combination with residual dipolar couplings to define the fold of cytochrome C' in the complete absence of NOE restraints. Very recently, this same group has determined the global fold of ubiquitin to 1.0 Å backbone RMSD (residues 1-71) with respect to the solution structure determined by conventional methods, using restraints derived solely from $N_{i+1}-H_{i+1}^N$, C'_i-N_{i+1} , $C'_i-H_{i+1}^N$, $C_i^\alpha-C'_i$, $C^\alpha-H^\alpha$ and $C^\alpha-C^\beta$ residual dipolar couplings in two independent tensor frames. Hus et al., *J. Am. Chem. Soc.* 123:1541-1542, 2001.

[0009] These methods of protein fold determination, however effective, have the major drawback of being difficult and time-consuming. Furthermore, the complexity of the calculations needed and the large number of data points makes determination of the global fold of large proteins difficult

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to obtain. This is due largely to the use of universally isotopically enriched material which yield split signals in the NMR spectrum, each of which need to be assigned before a structure determination can be commenced. Splitting of signals results in both more and weaker signals. This phenomenon causes overlap of signals and a far inferior signal-to-noise ratio, both of which make the assignment process more difficult and rule out automation of the process. Methods currently available therefore can provide accurate structures or provide some structural data relatively quickly and easily, but no methods for rapid determination of the global fold are available which can also achieve the degree of accuracy which is desired. The ability to automate the various steps in the process would be of great advantage in achieving both rapid and sufficiently accurate results, however this has not been possible using the available techniques.

SUMMARY OF THE INVENTION

[00010] This invention provides a method for rapidly determining the three-dimensional structure of a molecule having a peptidic sequence of three or greater amino acids, including large proteins of greater than 30 kilodaltons, 50 kilodaltons, or larger. The method overcomes what has been a fundamental problem in NMR spectroscopy and a barrier to obtaining useful structural information for a number of proteins.

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[00011] Generally, the method involves subjecting the peptide or protein molecule to NMR analysis, assigning the molecule by computer based on the NMR analysis and measuring residual dipolar couplings of the protein in two different partially aligned states. The protein or peptide may be, for example, dissolved in two different liquid crystalline solutions or mechanically aligned. Liquid crystalline solutions are well known in the art. Therefore, a skilled person can easily select a suitable medium to impart partial alignment to the protein of interest. The invention is contemplated for use with any medium (such as a solvent or solution) or mechanical means which imparts partial alignment to the protein to be analyzed, including any suitable liquid crystalline medium.

[00012] Using the magnitudes and orientations of the principal axes of the alignment tensors, the orientation of dipeptide fragments of the protein are calculated with a computational algorithm. As a first step, the peptide or protein molecule is substituted on the backbone with ^{13}C , ^{15}N , or both ^{13}C and ^{15}N and the $\text{C}\alpha$ position protons are optionally substituted with ^2H . Measurement in two different partially aligned states, each of which provides a different order in two dimensions, facilitates great improvement in the ease of fold calculations.

[00013] The ϕ, ψ angles for a first amino acid of the protein are varied computationally. The rigid-body orientation of the first amino acid and a second amino acid adjacent in the peptidic sequence to the first amino acid are minimized with respect to both tensor frames simultaneously and the minimum difference between measured and calculated dipolar couplings

for each of the first and second amino acids are calculated. The ϕ, ψ angles and hence the orientation of the dipeptide fragment of the larger peptide or protein sequence (composed of the first and second amino acids) are derived from these calculated dipolar couplings. The steps are repeated for each sequential dipeptide fragment of the larger molecule to obtain the structure of the peptide or protein backbone. The same steps also may be repeated for each of the secondary structural elements as well. The result is global fold determination in days instead of months if this process is automated.

[00014] Accordingly, the invention provides a method for determining the global fold of a peptidic molecule having a sequence of three or greater amino acids which comprises the steps of (a) providing the molecule in a form which is substituted on the backbone with an isotope selected from the group consisting of ^{13}C , ^{15}N , and both ^{13}C and ^{15}N ; (b) subjecting the substituted molecule to NMR analysis in a non-aligned medium; (c) assigning the molecule by computer based on the NMR analysis; (d) placing the molecule in a first state of partial alignment and measuring residual dipolar couplings for the molecule in the first state of partial alignment, wherein the magnitudes and orientations of the principle axes of the alignment tensors for the first state of partial alignment are known or obtained; (e) placing the molecule in a second state of partial alignment and measuring residual dipolar couplings for the molecule in the second state of partial alignment, wherein the magnitudes and orientations of the principle axes of the alignment tensors for the second

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state of partial alignment are known or obtained; (f) varying computationally by increments the ϕ, ψ angles for a first amino acid of the molecule; (g) minimizing the rigid-body orientation of the first amino acid and a second amino acid adjacent in the peptidic sequence to the first amino acid with respect to both tensor frames simultaneously; (h) calculating the minimum difference between measured and calculated dipolar couplings for each of the first and second amino acids; (i) deriving the ϕ, ψ angles and orientation of the dipeptide fragment composed of the first and second amino acids; and (j) repeating steps (f)-(i) for each sequential dipeptide fragment of the molecule to obtain a global fold of the peptidic molecule. The method may further comprise repeating steps (f)-(i) for at least one secondary structural element. The invention also provides methods in which the peptidic molecule is further isotopically substituted with ^2H at the $\text{C}\alpha$ position protons.

[00015] In a further embodiment, the invention provides methods wherein the residual dipolar couplings for the molecule are measured in step (d) above in at least two different media which impart a weak alignment to the molecule. The media may be liquid crystalline solutions.

[00016] In yet a further embodiment, the invention provides methods which further comprise additionally performing steps (b) through (j) using the peptidic molecule which has been universally isotopically substituted in one or more species of amino acid, or in one species of amino acid.

[00017] In yet a further embodiment, the invention provides a method as described above which further comprises refining the

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global fold of the peptidic molecule by including data concerning interatom distances, for example NOE data.

[00018] In yet a further embodiment, the methods use measurement of at least three residual dipolar couplings for each state of partial alignment, for example three, four, five, or more than five residual dipolar couplings.

[00019] In yet a further embodiment, the invention provides a structural map obtained by any of the methods described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[00020] Figure 1 shows NMR spectral data using conventional universal isotopic labeling of a protein (1A) and backbone only isotopic labeling of β -lactamase, a protein approximately twice the size (1B).

[00021] Figure 2 is a schematic diagram of a protein fragment showing ϕ and ψ .

[00022] Figure 3 is schematic diagram illustrating the refinement of a protein structure.

[00023] Figure 4 is a group of contour maps providing examples of residues in β -pleated sheet, α -helix and loop regions of a protein.

[00024] Figure 5 is a group of contour maps providing data for selected residues of ubiquitin.

[00025] Figure 6 is a set of stereoscopic images of the global fold of residues 3-73 of ubiquitin. Figure 6A shows the results from an intermediary calculation while Figure 6B shows the results from the refined calculation.

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[00026] Figure 7 is a flow chart showing some of the steps disclosed for the method of obtaining global fold data.

[00027] Figure 8 is a schematic diagram demonstrating calculation of a bond angle.

[00028] Figure 9 is a schematic diagram showing measurement of dipolar couplings assisted by weakly aligning the molecule under study.

[00029] Figure 10 is a set of contour maps for two residues of ubiquitin as indicated.

[00030] Figure 11 is a stereoscopic diagram providing the global fold of α -helix (24-34) of ubiquitin, as determined by the inventive methods and by X-ray crystallography.

[00031] Figure 12 is a stereoscopic diagram providing the global fold of β -sheet (3-15) of ubiquitin, as determined by the inventive methods and by X-ray crystallography.

[00032] Figure 13 is a stereoscopic diagram providing the global fold of ubiquitin, as determined by the inventive methods and by X-ray crystallography.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[00033] The methods described here enable automation of the assignment process and the rapid calculation of the structure of the protein backbone. This reduces the time taken for structural analysis of a protein by NMR from years or months to only a few days. Provided the protein to be studied can be purified, structural information can be achieved very rapidly for proteins of any size and type, and from any source.

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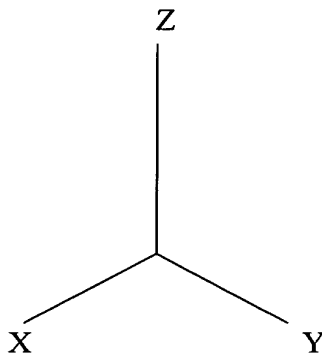
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[00034] Incorporation of the stable isotopes ^{13}C , ^{15}N into the backbone of the protein, but not the amino acid side chains (and optionally ^2H in the $\text{C}\alpha$ position protons) eliminates the causes of poor spectral quality normally seen in NMR spectra of proteins, greatly simplifies assignment and allows the NMR techniques to focus on signals relevant to determining the global fold. See Figure 1. Such backbone labeling of peptides and proteins may be achieved using the methods disclosed in United States Patent No. 6,111,066, the disclosures of which are hereby incorporated by reference. Generally, the number of signals seen is reduced by a factor of four, while multiple signals are condensed into sharp singlets. Both these factors increase the signal to noise ratio and greatly reduce signal overlap. The greater quality of the spectra allows assignment of signals to be automated, which previously had not been possible. The inventive methods also allow very rapid protein global fold determination because the number of residual dipolar coupling restraints can be reduced while still providing good accuracy in global fold determination. These assigned signals can be used to calculate the global fold of the protein according to the methods of this invention.

[00035] Although the methods of this invention are suitable for the determination of structural information of any peptidic molecule of three or more amino acids in length, and therefore encompass both proteins and peptides, the description, for simplicity, will refer only to proteins. It is understood that the term "protein," in this application, refers to any peptidic molecule of three or greater amino

[00036] According to this invention, the NMR spectrum of a protein is acquired with the protein in a partially aligned state. When non-spherical macromolecules, such as proteins, are dissolved in a dilute liquid-crystalline medium, for example, there is a small but significant and measurable tendency for the molecules to adopt a particular overall orientation (alignment) in the solution relative to the tensor frame of the particular liquid crystalline medium. As a result of this weak orientational tendency, residual dipolar couplings are discernable from the NMR spectrum of the macromolecule. Alternatively, the molecules can be dissolved in any solvent or solution which imparts a weak alignment to them. For example, it is known in the art to place proteins in a solution containing phage for NMR analysis. In addition, the molecules may be mechanically aligned, for example by dissolving in polyacrylamide gel or using physical pressure alignment. Although the description and examples below, for the sake of simplicity, refer to partial alignment in liquid crystalline solution, it is understood that any of the described methods alternatively may use partial alignment of the molecules by any suitable method, including partial alignment in other types of solutions or mechanically.

[00037] For a given chemical bond, for example the bond between a proton (^1H) and a carbon (^{13}C) nucleus, the size of the measured dipolar coupling depends on the orientation of the bond with respect to a coordinate frame which is sometimes referred to as the "principal axis system of the alignment tensor" or "tensor frame." Although the derivation of this tensor is complex, in its simplest form it is cartesian coordinate system with the usual x, y and z axes:



The dipolar coupling depends upon orientation according to Equation I:

$$D_{PQ}(\theta, \phi) = S \frac{\mu_o}{4\pi} \gamma_P \gamma_Q h \left[A_a (3\cos^2\theta - 1) + \frac{3}{2} A_r \sin^2\theta \cos 2\phi \right] / 4\pi^2 r_{PQ}^3$$

[00038] Calculation of θ and ϕ for each bond is performed by measuring the dipolar coupling by NMR and solving the above equation. However, due to the functional form of the equation, there are a minimum of eight solutions (*i.e.*, eight θ, ϕ pairs) resulting from each dipolar coupling. Thus, for each bond there is an eight-fold ambiguity in its calculated position. The ambiguity in the total structure of a molecule increases exponentially (to the power of the number of bonds in the molecule). For a peptide or protein, this quickly results in a prohibitively large number of possible solutions. One way to resolve the ambiguity inherent in this equation is to measure dipolar coupling in a second tensor frame that orients the molecule differently. The molecule is therefore oriented along a second principal axis system non-coincident with the first. Under these circumstances, provided a sufficient number of dipolar couplings can be measured for a given molecular fragment, there is a single solution to the above Equation I. The bond angle is thus unambiguously determined. Fewer restraints are required to obtain the structure than methods using NOEs alone, so the method is able to provide results more quickly and easily. Without wishing to be bound by theory, only three residual dipolar couplings per tensor frame are required to obtain an accurate, unambiguous structure. It is contemplated that at least three

residual dipolar couplings are measured per tensor frame, for example, 3, 4, 5, or more than 5 residual dipolar couplings.

[00039] By walking along the protein backbone, two residues at a time, the relative orientations of each residue pair are sequentially determined. Thus, measuring the residual dipolar coupling data for each dipeptide fragment of the protein in two independent tensor frames (e.g., in two different liquid crystalline solutions) allows one to calculate the bond angles of each of the various bonds in the protein chain so that the global fold is obtained. See Figure 2.

[00040] Mapping the structure of a protein using backbone dipolar couplings is achieved by varying, by computer, ϕ and ψ bond angles by increments from 0° through 360° . Fifteen degree increments are sufficient, however it is contemplated that increments of about 15 to about 5 are preferred, for example, 12 degrees, 10 degrees, 8 degrees or 5 degrees. Three dimensional ϕ, ψ potential surfaces may be calculated using a suitable commercially available program such as, for example, XPLOR. Advantageously, an extended structure for the protein may be generated by setting all ϕ, ψ angles to 180° (with the exception of ϕ for prolines). Groups of three residues ($i-1, i, i+1$) then may be considered stepwise from the COOH terminus, with the sidechain of residue i truncated at C^β . The values of ϕ and ψ for residue i may be varied independently through 360° in 15° increments, resulting a two dimensional grid of points. At each point, a rigid body minimization is performed on the tripeptide fragment to minimize the difference between experimental and theoretical residual dipolar couplings $N_i-H_i^N$, $N_{i+1}-H_{i+1}$, $C_i^\alpha-H_i^\alpha$, $H_i^N-C'_{i-1}$,

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$H_{i+1}^N-C'_i$ with respect to two sets of external Cartesian axes whose relative orientation is defined by the two tensor frame orientations. To overcome the effects of local minima during the minimization procedure, the rigid body minimization may be performed multiple times, for example about 5 to about 20 times or preferably about 10 times, at each grid point, starting with randomized values of the three Euler angles that describe the orientation of the tripeptide fragment in the tensor frames. The axial A_α , component and rhombicity R (Bax, *Science* 278:1111-1114, 1997) in each tensor frame may be taken from published information or may be determined according to any known and convenient method. Force constants are used for $N-H^N$, $C^\alpha-H^\alpha$ and H^N-C' residual dipolar couplings, respectively. See Tjandra et al., *Nature Struct. Biol.* 4:732, 1997. In addition, a weak repulsive van der Waals term was included to account for steric clashes involving the C^β atom of residue i . Use of such a van der Waals term is considered standard practice by those in the art and is well understood. Therefore, skilled persons can readily include a suitable van der Waals term. Commercially available programs generally include an option to use a van der Waals term. The resulting potential surfaces are contoured automatically using, for example, any conventional contour plotting algorithm such as gnuplot, which is available free over the internet, or any other convenient computer software. Structural elements determined by this method may be refined, if necessary, using a limited number of NOEs and assembled into a final structure. See Figure 3.

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[00041] Residual dipolar coupling data, measured in two independent tensor frames (two different states of partial alignment) for a given dipeptide fragment in a protein depend on five parameters: namely the backbone torsion angles ϕ and ψ and the three Euler angles representing the orientation of the fragment in the tensor frame (assuming a fixed orientation of the peptide plane). With two tensors, the orientation of a chiral motif is completely defined. Ramirez et al., *J. Am. Chem. Soc.* 120:9106-9107, 1998; Wang et al., *J. Am. Chem. Soc.* 120:7385-7386, 1998; al-Hashimi et al., *J. Magn. Reson.* 143:402-406, 2000; Hus et al., *J. Am. Chem. Soc.* 123:1541-1542, 2001. The inventive method takes advantage of this to determine the global fold of a protein. To determine the structure of the complete protein in the minimum time, it is advantageous to use at least the three largest residual dipolar couplings that can readily be measured in proteins ($N_i-H_i^N$, $C_i\alpha-H_i^\alpha$ and $H_i^N-C'_{i-1}$). These couplings can be determined to reasonable accuracy using an 'IPAP' type approach without recourse to time-consuming J-modulation methods. Ottinger et al., *J. Magn. Reson.* 131:373-378, 1998; Lerche et al., *J. Magn. Reson.* 140:259-263, 1999; Tjandra and Bax, *J. Magn. Reson.* 124:513-515, 1997.

[00042] Tensor orientations of a dilute liquid crystalline solution or any other solution are readily available if the structure of a molecular fragment is known. In the absence of a known structure, models obtained from idealized secondary structure elements may be used in some cases, however large errors for the dipolar couplings have to be used to account for deviation of the actual structure of the molecular

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fragment from the idealized structure. Fowler et al. *Biophys. J.* 78:2827Pos, 2000. In unfavorable cases, where there is a significant deviation of the rigid structure used as a model and the actual structure of the molecular fragment, it is not possible to obtain any order tensor orientation from idealized structures. To work around this problem, a simulated annealing approach may be used to simultaneously refine an idealized starting structure to obtain the relative orientation of the order tensor frames. For example, the relative orientation of two tensor frames may be obtained using only H^N-N and $C\alpha H\alpha$ residual dipolar one-bond couplings from two different alignment media and an idealized α -helix as starting structure.

[00043] Having the tensor parameters, global fold determination according to this invention first involves the derivation of ϕ, ψ values for each dipeptide fragment that is compatible with the measured dipolar couplings in each tensor frame. By analogy with the work of Wang et al., *J. Am. Chem. Soc.* 120:7385-7386, 1998, this can be achieved by use of a grid-search over ϕ and ψ , while simultaneously optimizing the orientation of the dipeptide fragment relative to the two alignment tensors that gives the best fit between predicted and measured couplings. This can be achieved by incorporating residual dipolar couplings as pseudo-energy terms in a conventional energy minimization protocol. See Clore et al., *J. Magn. Reson.* 131:159-162, 1998; Clore et al., *J. Magn. Reson.* 133:216-221, 1998; Clore et al., *J. Am. Chem. Soc.* 121:6513-6514, 1999, the disclosures of which are hereby incorporated by reference. In addition, a van der Waals'

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[00044] The minimum difference between measured and calculated dipolar couplings for each of these two amino acid fragments gives experimentally derived ϕ, ψ angles and the orientation of the dipeptide fragment. Generally, unique values of the ϕ, ψ angles are found for regions of secondary structure in the protein, whereas multiple solutions may be found for loop regions (compare Figures 4A-4D with 4E). Multiple solutions for loop regions are not unexpected since loop regions usually are mobile in proteins. The only other angle defining protein backbone conformation, ω , is invariably 180° due to planarity of the peptide group.

[00045] In most cases, a single pair of ϕ, ψ values is compatible with the measured couplings in accord with theoretical predictions is obtained (see Figures 5A and 5B). However, in certain instances, two or more minima can be observed on the ϕ, ψ surface that have very similar energies (Figure 5C). Such ambiguities can arise from a number of sources, including absent data for certain residues, coincidences between bond vector and tensor orientations or limitations in the accuracy of experimental residual dipolar couplings. These ambiguities usually can be resolved by

consideration of the known sensitivity of the $C\alpha$ chemical shift of a given residue to the conformation about ϕ and ψ . See Beger and Bolton, *J. Biol. NMR* 10:129-142, 1997, the disclosures of which are hereby incorporated by reference. In the case of ubiquitin, using the residual dipolar couplings described by Ottiger and Bax (*J. Am. Chem. Soc.* 120:12334-12341, 1998), 40 ϕ, ψ pairs could be determined unambiguously, and a further 23 pairs could be determined by consideration of $C\alpha$ chemical shifts. A number of ϕ, ψ pairs could not be determined unambiguously due to insufficient data (residues M1, Q2, T9, I36, P37, P38, D52, R74) or lack of chirality (glycine residues). In general, the residues having insufficient data were located in loop regions of the protein. The relevant data are summarised in Table I, below.

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Table I. Values of ϕ and ψ Derived from Residual Dipolar Couplings in Ubiquitin.

| Residue | Dihedral angles | | Residue | Dihedral angles | | Residue | Dihedral angles | | Residue | Dihedral angles | |
|---------|-----------------|--------|---------|-----------------|--------|---------|-----------------|--------|---------|-----------------|--------|
| | ϕ | ψ | | ϕ | ψ | | ϕ | ψ | | ϕ | ψ |
| M1 | n/a | n/a | S20*\$ | -80 | 180 | D39\$ | -60 | 120 | D58* | -50 | -30 |
| Q2 | n/a | n/a | D21 | -80 | 150 | Q40 | -100 | -20 | Y59 | -100 | 0 |
| I3 | n/a | n/a | T22* | -80 | 170 | Q41 | -90 | 130 | N60 | 60 | 45 |
| F4* | -120 | 140 | I23* | -70 | -60 | R42 | -120 | 120 | I61 | -80 | 110 |
| V5 | -130 | 110 | E24* | -80 | -60 | L43 | -100 | 130 | Q62 | -100 | 160 |
| K6* | -100 | 130 | N25 | -70 | -40 | I44* | -120 | 150 | K63 | -50 | 140 |
| T7 | -100 | 160 | V26 | -60 | -60 | F45 | -150 | 120 | E64 | 70 | 30 |
| L8* | -100 | -30 | K27 | -50 | -40 | A46 | 70 | 60 | S65 | -90 | 150 |
| T9 | m | m | A28 | -60 | -30 | G47 | m | m | T66 | -110 | 130 |
| G10 | m | m | K29 | -70 | -40 | K48* | -120 | 150 | L67 | -120 | 160 |
| K11 | -90 | 140 | I30* | -80 | -40 | Q49* | -85 | 140 | H68 | -120 | 150 |
| T12* | -120 | 130 | Q31 | -60 | -40 | L50* | -80 | 140 | L69* | -100 | 120 |
| I13* | -110 | 120 | D32* | -70 | -40 | E51 | -90 | 140 | V70 | -120 | 150 |
| T14 | -105 | 130 | K33* | -80 | -60 | D52 | m | m | L71 | -90 | 140 |
| L15* | -120 | 160 | E34*\$ | -110 | 180 | G53 | m | m | R72 | -100 | 120 |
| E16 | -100 | 120 | G35*\$ | -80 | -30 | R54 | -110 | 160 | L73 | -120 | 110 |
| V17 | -135 | 170 | I36 | m | m | T55* | -100 | 160 | R74 | n/a | n/a |
| E18 | -110 | 130 | P37 | - | m | L56 | -60 | -40 | G75 | n/a | n/a |
| P19 | - | -50 | P38 | - | m | S57* | -60 | -30 | G76 | n/a | n/a |

* -- Value determined from two or more minima on the potential surface, resolved by consideration of C α chemical shift.

m -- Multiple minima on ϕ, ψ surface, not resolvable by consideration of C α chemical shift.

\$ -- Value inconsistent with crystal structure.

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[00046] In principle, knowledge of all ϕ, ψ pairs is sufficient to determine the global fold of the protein. However, in structure calculations using the subset of ϕ, ψ pairs given in Table I, global fold could not be obtained due to lack of dihedral angle data for loop regions in the protein.

[00047] Since residual dipolar coupling data contain no translational information, there are insufficient structural restraints from these dipolar coupling data alone to generate a unique fold. Therefore, a limited number of NOE distance restraints, namely H^N-H^N NOEs, in addition to the dihedral restraints listed in Table I, were incorporated in simulated annealing calculations as described in Example 1. During the course of these calculations, several $H_i^N-H_{i+1}^N$ NOEs restraint violations greater than 0.5 Å (S20 - D21, D32 - G35, E34 - D35, D39 - Q40) were noted, with corresponding ϕ, ψ restraint violations. For each of these violations, comparison of the restrained values of ϕ, ψ (Table I) with the corresponding values in the crystal structure, indicated that for certain residues (S20, E34, G35 and D39), incorrect ϕ, ψ values were predicted by the residual dipolar couplings.

[00048] In the case of S20 (Figure 5D), a broad minimum was observed at $\phi, \psi = -80^\circ, 180^\circ$, consistent with the $C\alpha$ chemical shift. However, the "correct" minimum is located at $\phi, \psi \sim -80^\circ, 0^\circ$. This can be observed in Figure 5D, but is 0.7 kcal/mol above the global minimum. The situation is similar for E34 (Figure 5E), although in this case the "correct" minimum at about $\phi, \psi -120^\circ, 0^\circ$ is only 0.16 kcal/mol above the global minimum. For G35, the $C\alpha$ chemical shift is consistent with an α -helical configuration, but the potential surface is

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symmetric about ϕ and ψ due to the lack of chirality information about $C\alpha$ (Figure 5F). Finally, for D39, the $C\alpha$ chemical shift predicts correctly an α -helical configuration, yet two very closely spaced minima are observed near $\phi, \psi = -40^\circ, 120^\circ$ (Figure 5G).

[00049] While these discrepancies were clearly detectable from dihedral and NOE restraint violations, the goal of this invention is to generate accurate global folds for proteins without any reference to crystal structure data. The structure calculation protocol therefore was repeated according to the invention, with deletion of the ϕ, ψ restraints corresponding to these violations. This structure calculation (determined from $N_i-H_i^N$, $C_i^\alpha-H_i^\alpha$ and $H_i^N-C'_{i-1}$, residual dipolar coupling and H^N-H^N NOE restraints) gave rise to a global fold whose backbone RMSD for the lowest energy structure of 20 structures was 2.28Å with respect to the crystal structure (residues 3-73, Figure 6 and Table II).

Table II. Structure Quality for the Global Fold of Ubiquitin.

| | initial | refined |
|--|------------|---------|
| Measures of Structure Quality | | |
| E_{L-J} (kCal mol ⁻¹) ¹ | 97(+81-36) | 58±9 |
| Coordinate Precisions ² (Å) | | |
| Backbone rrnsd of Lowest Energy | 1.6 | 0.8 |
| Structure ³ (Å) | 2.28 | 1.8 |

¹ E_{L-J} = average Lennard - Jones energy calculated using XPLOR **** protein parameters.

² Average rms difference between the 20 final simulated annealing structures and the mean coordinates. The values given relate to residues 3-73.

³ The values given relate to residues 3-73.

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[00050] Inclusion of residual dipolar coupling data in the form of biharmonic ϕ, ψ restraints in the manner described above does not directly include information on the orientation of each dipeptide fragment with respect to the tensor frames. This information is of course available from residual dipolar coupling measurements, and can be re-introduced by direct refinement of the global fold obtained above against residual dipolar couplings, in a manner analogous to that described by Tjandra et al. (*Nature Str. Biol.* 4:732-738, 1997), but with fixed, rather than floating tensor-frame orientations.

[00051] In the initial stages of the simulated annealing protocol, strong biharmonic restraints were applied corresponding to known values of ϕ, ψ , which were slowly reduced to zero during the cooling stage with concomitant increase in direct residual dipolar coupling restraints (see Example 1). This refinement protocol gave rise to a global fold whose backbone RMS for the lowest energy structure of 20 structures was 1.8 Å with respect to the crystal structure (residues 3-73, Figure 6 and Table II).

[00052] One unexpected observation was that the five residual dipolar couplings $N_i-H_i^N$, $N_{i+1}-H_{i+1}^N$, $C_i^\alpha-H_i^\alpha$, $H_i^N-C'_{i-1}$, $H_{i+1}^N-C'_i$ observed in a dipeptide fragment can, for certain residues, be consistent with values of ϕ and ψ that are clearly incorrect, based on the crystal structure of ubiquitin. In this example, such inconsistencies were obvious from NOE and dihedral restraint violations and thus would be detected even in cases where a crystal structure was not available.

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[00053] In the case of S20 and E34, the correct ϕ, ψ values correspond to a minimum on the potential surface, but not the global minimum. In both of these cases, the $C\alpha$ chemical shift predicts incorrectly that the global minimum ϕ, ψ values are the correct ones. Notably, the correct ϕ, ψ values for S20 and E34 are predicted correctly if additional dipolar restraints between $C\alpha_i-C'_i$ and $N_{i+1}-C'_i$ are included in the gridsearch procedure, suggesting that the discrepancies are due to limited data. In contrast, ϕ, ψ values for D39 are not correctly predicted even with these additional residual dipolar restraints. In the study of Wand et al. (*Biochemistry* 35:6116-6125, 1996) the S^2 value for D39 is reported to be very low (0.56), on the basis of $^{13}C\alpha$ relaxation data. An implicit assumption in the present work is that all residues possess a uniform S^2 value.

[00054] Despite the lack of ϕ, ψ restraints for certain residues due to the factors discussed above, it is clear from the results in Table II that it is possible to determine the global fold of ubiquitin to an accuracy of approximately 1.8 Å backbone RMSD using an average of six dipolar coupling restraints (three in each of two tensor frames) and approximately 2 NOE restraints per residue. This degree of accuracy is more than adequate for use in databases of structural motifs, and provides a method for the determination of protein global folds using conformational restraints derived from backbone atoms only. This method therefore obviates the need to undertake the time-consuming task of sidechain assignment, resulting in an enormous time and labor savings. In contrast to previously available methods, highly accurate

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protein structural information can be obtained rapidly using a far smaller data set. The small size of the data set used with the inventive methods is an important factor, but in addition, the precise nature of the data obtainable with appropriately isotopically substituted (backbone labeled) proteins allows automation of the process, which was not feasible before. Thus, the methods described here represent an advance in the art which allows both rapid and accurate determination of protein global fold.

[00055] Whether a particular level of accuracy can be achieved in a particular case depends on a number of factors. First, the $C_i^\alpha-H_i^\alpha$ residual dipolar coupling is very important since it provides information on chirality, and the ϕ, ψ potential surface displays C_2 symmetry in its absence. Wang et al. *J. Am. Chem. Soc.* 120:7385-7386, 1998. This coupling is difficult to measure in larger proteins due to the efficient dipolar relaxation between directly bonded $C^\alpha-H^\alpha$ pairs. When these coupling data are not available, however, the $C_i^\alpha-C^\beta$ residual dipolar coupling can be used as an alternative source of chirality information. Advantageously, the $C_i^\alpha-C^\beta$ residual dipolar coupling can be measured without the need to assign the C^β atoms.

[00056] Second, in proteins containing a high α -helical content, the long-range H^N-H^N NOE restraints required for packing of secondary structural elements may not be available in all cases, and backbone-sidechain or sidechain-sidechain NOEs may be important for determination of global folds. A small number of such NOEs is sufficient for the success of the inventive methods, however. For example, parts of the global

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fold of *E. coli* acyl carrier protein were derived using residual dipolar coupling measurements together with one backbone-backbone, but the technique also required four backbone-sidechain NOES.

[00057] The global fold of *Rhizobium leguminosarum* NodF protein was determined with low resolution using five backbone-backbone NOES and additional data. Fowler et al., *J. Mol. Biol.* 304:447-460, 2000. Clearly, the use of NOEs involving sidechain atoms requires the assignment of the sidechain atoms, which significantly increases the time required to derive the global fold. However, since the number of NOEs required is small in the inventive methods, the effort required for assignment can be reduced by careful choice of isotopic substitution strategies based upon residue type. Therefore, the methods include use of proteins which may be universally isotopically labeled with ^{13}C , ^{15}N or both ^{13}C and ^{15}N in one or more species of amino acid (one residue type, for example, leucine, valine or isoleucine). Proteins which are universally isotopically labeled with ^{13}C , ^{15}N or both ^{13}C and ^{15}N on only a single amino acid in the sequence, or two or more amino acids, also are contemplated for use with the invention. Any convenient method may be used for isotopic substitution of proteins and peptides, and such methods are known to those of skill in the art.

[00058] To summarize, the general protocol (see flowchart in Figure 7) for obtaining a global fold (defined as the complete three dimensional structure of the protein backbone) according to the invention is as follows:

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1. A protein is selected for study and synthesized with backbone only isotopic substitution with ^{13}C , ^{15}N , or both ^{13}C and ^{15}N and optional isotopic substitution with ^2H at the $\text{C}\alpha$ position protons. This isotopic substitution allows the determination of global folds of the largest possible proteins due to improvement in the NMR data that are thereby obtained.
2. The protein is subjected to NMR spectroscopic analysis in an aqueous (non-aligned) solution. See Figures 8 and 9. The term J is measured as the distance between split peaks in non-aligned medium. This distance represents both D and J when measured in aligned media. D can be calculated from measurement in aligned and non-aligned media (see Figure 9). The equation then can be solved for θ , however there is more than one solution to the equation.
3. The protein is assigned by computer, taking advantage of the substantial sensitivity and resolution gain available from the backbone labeling protocol. Assignment is the identification of which signal in the NMR data comes from which atom in the molecule being assayed. Because the NMR data used is very clean and precise, with sharp peaks and little splitting, this process is much simpler and can be performed by computer, unlike previous methods. The method may involve identification of the N-terminal nitrogen ($-\text{NH}_2$), then the adjacent $\text{C}\alpha$, then the next atom (the carbonyl carbon) and so on, walking along the backbone of the peptide sequence. Ikura et al., *Biochemistry* 29:4659-4667, 1990.

4. Residual dipolar couplings are measured for the protein in two different partially aligned states, for example in two different liquid crystalline solutions. This allows unambiguous solution of Equation I to obtain a calculated θ angle for each bond. Three, four, five or more residual dipolar couplings may be measured in each partially aligned state (tensor frame). Preferably, three residual dipolar couplings are sufficient.
5. The magnitudes and orientations of the principal axes of the alignment tensors are obtained for each partial alignment (e.g. each liquid crystalline solvent) by using any suitable conventional method, such as, for example, matrix diagonalization or grid-search or from published data.
6. Using computational algorithms known in the art, the ϕ, ψ angles for a given amino acid are independently varied, in 15-5 degree steps. The rigid-body orientation of this amino-acid and the next amino acid in the polypeptide chain is minimized with respect to both tensor frames simultaneously.
7. Using the ϕ, ψ angles obtained for the secondary structural elements, a three dimensional structure (global fold) is built for the protein, defining these secondary structural elements.
8. The global fold of the protein is refined from the initial structure obtained in step 7 by further use of residual dipolar couplings to align secondary structural elements. Distance restraints derived from NH-NH NOEs also may be employed for this purpose.

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EXAMPLES

Example 1. Derivation of Tensor Frame Orientations.

[00059] Experimental residual dipolar coupling data in two tensor frames were taken directly from Ottiger and Bax, *J. Am. Chem. Soc.* 120:12334-12341, 1998. H^N-H^N NOEs restraints were computed from the crystal structure of ubiquitin using a simple distance matrix approach and including all H^N-H^N distances less than 5 Å.

[00060] The relative orientations of the tensor frames were calculated using a simulated annealing approach. Each of two idealized α -helices ($\phi=-57^\circ$, $\phi=-47^\circ$) representing fragments K29-E34 (fragment 1) and N25-I30 (fragment 2) of the long ubiquitin helix were used as starting structures for molecular dynamics simulation. Experimentally obtained one-bond H^N-N and $C\alpha H\alpha$ dipolar couplings from two different alignment media and simulated H^N-N NOE-data were used as restraints in XPLOR simulated annealing refinement protocols. See Ottinger and Bax, *J. Am. Chem. Soc.* 120:12334-12341, 1998 and Brünger, XPLOR, version 3.1: A System For X-ray Crystallography and NMR, Yale University Press, New Haven, CT, 1987, the disclosures of which are hereby incorporated. The first alignment tensor was fixed in this protocol, while the second tensor and the helical fragment were allowed to reorient in the course of the calculation. By altering the structure and reorientation of the second tensor the H^N-N NOEs and the dipolar coupling restraints were satisfied.

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Example 2. Determination of ϕ, ψ Values for Residue Pairs.

[00062] Three dimensional ϕ, ψ potential surfaces were calculated for ubiquitin using XPLOR version 3.851. First, an extended structure for ubiquitin was generated by setting all ϕ, ψ angles to 180° (with the exception of ϕ for prolines).

Groups of three residues ($i-1$, i , $i+1$) were then considered stepwise from the COOH terminus, with the sidechain of residue i truncated at C^β . The values of ϕ and ψ for residue i were each varied independently through 360° in 15° increments, resulting a two dimensional grid of 576 points as in Figure 4. At each point, a rigid body minimization was performed on the tripeptide fragment to minimize the difference between experimental and theoretical residual dipolar couplings $N_i-H_i^N$, $N_{i+1}-H_{i+1}$, $C_i^\alpha-H_i^\alpha$, $H_i^N-C'_{i-1}$, $H_{i+1}^N-C'_i$ with respect to two sets of external cartesian axes whose relative orientation is defined by the two tensor frame orientations determined above. To overcome the effects of local minima during the minimization procedure, the rigid body minimization was performed ten times at each grid point, starting with randomized values of the three Euler angles that describe the orientation of the tripeptide fragment in the tensor frames. See Figure 10. The axial A_α component and rhombicity R in each tensor frame was taken from Ottiger and Bax, *J. Am. Chem. Soc.* 120:12334-12341, 1998, the disclosures of which are hereby incorporated by reference. Force constants of 0.5, 0.247, and 1.61 kcal mol⁻¹ HZ⁻² were used for $N-H^N$, $C^\alpha-H^\alpha$ and H^N-C' residual dipolar couplings, respectively. In addition, a weak repulsive van der Waals term was included to account for steric clashes involving the C^β atom of residue i . The resulting potential surfaces were contoured automatically using a script written for the program Maple, although any other suitable and convenient program known in the art may be used. See Figures 11 and 12.

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[00063] The global fold of ubiquitin was determined with H^N-H^N NOE restraints and residual dipolar coupling-derived dihedral restraints using the XPLOR simulated annealing script (sa.inp) (Brünger, XPLOR, version 3.1: A System for X-ray Crystallography and NMR; Yale University Press, New Haven, CT, 1987) with the following differences. Restraints corresponding to values of ϕ and ψ for which unique solutions exist could be derived from the plots derived in the gridsearch procedure above. See Figure 4. Together with consideration of $C\alpha$ chemical shifts as necessary, they were applied as standard biharmonic dihedral restraints. An initial force constant of 200.0 kcal mol⁻¹ rad⁻² was used, rising to 500.0 kcal mol⁻¹ rad⁻² at the end of each simulated annealing run. A total of 6000 molecular dynamics steps of 5.0 fs were computed at a temperature of 1000 K, followed by 3000 cooling steps of 5.0 fs to a final temperature of 100 K. This standard type of method removes a thermal element from the structure in which the intramolecular movements are reduced to near zero. Twenty structures were calculated from an initial starting structure whose ϕ, ψ angles were set to those defined by the residual dipolar coupling data where unique values were available. Where the values of ϕ, ψ were not available from the residual dipolar coupling data alone, or were ambiguous, these were set to 180° in the starting structure.

[00064] Global fold refinement was performed with the lowest energy structure from the previous set of calculations as

input. This initial structure was refined against residual N-H^N, C α -H α and H^N-C' dipolar coupling and H^N NOE data using the XPLOR script sa.inp (Brünger, XPLOR, version 3.1: A System for X-ray Crystallography and NMR; Yale University Press, New Haven, CT, 1987) with the following differences. In the high temperature phase involving 3000 MD steps of 5 fs at 500 K, dihedral angle restraints were applied as in the previous global fold determination, but with a force constant of 100 kcal mol⁻¹ rad⁻². In addition, weak N-H^N, C α -H α and H^N-C' residual dipolar coupling restraints were initially applied with a force constant of 0.02 kcal mol⁻¹ Hz⁻², using two external cartesian axes whose relative orientation was defined by the two tensor frame orientations determined in Example 1. During the cooling phase of 6000 steps of 1 fs, the dihedral force constant was reduced to zero at the end of the cooling period, with concomitant increase of the residual dipolar coupling force constant to 0.5 kcal mol⁻¹ Hz⁻². Twenty structures were calculated from the initial starting structure. The final structure (structural map) is shown in Figure 13.

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